FORM PTO-1390 (REV 11-98) U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: Bayer 10,184-KGB

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/341227

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP 97/07306	24 December 1997 (24.12.97)	08 January 1997(08.01.97)

TITLE OF INVENTION: ELECTROKINETIC SAMPLE PREPARATION

APPLICANT(S) FOR DO/EO/US: SEE ATTACHED "APPENDIX"

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    a) PCT/RO/101 - PCT Request (in German)
    b) PCT/IPEA/409 - International Preliminary Examination Report (in German)
    c) WO 98/30571 - World Publication - (in German)
    d) PCT/ISA/210 - International Search Report - (in English)(attached to published application)
    e) Five sheets of drawings (Figures 1-8)

U.S. APPLICATION NO. (if known, see 37 CFR 1.)	INTERNATIONAL APPLICATION NO	09 Rec'd PCT/PTO 07 JUL 1999	ATTORNEY'S DOCKET NUMBER
09734122	PCT/EP97/07306		Bayer 10,184-KGB

	CALCULATIONS	PTO USE ONLY
17. ☒ The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$760.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	12 - 20 =	0	X **$18.00**	$ 0	
Independent claims	1 - 3 =	0	X **$78.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$840.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$840.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$840.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$	
				Amount to be: refunded	$
				charged	$840.00

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 02-1445 in the amount of $840.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-1445. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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27,552
REGISTRATION NUMBER

Bayer 10,184-KGB
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	HANSJÖRG DÜRR, ULF BRÜGGEMEIER, KARSTEN DIERKSEN, HANS-ROBERT HEHNEN, RAINER NEUMANN AND EBERHARD KUCKERT
Serial No.	:	TBA
Filed	:	Herewith
For	:	ELECTROKINETIC SAMPLE PREPARATION
Group Art Unit	:	TBA
Examiner	:	TBA

July 7, 1999

Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, kindly amend the above-identified application as follows:

IN THE CLAIMS

Please amend the claims as follows:

Claim 1, lines 1 and 2, delete "characterized in that" and replace with --wherein--.

Claim 2, line 1, delete "characterized in that" and replace with --wherein--.

Claim 3, line 1, delete "Claims 1 and 2" and insert --Claim 1--.

Claim 4, line 1, delete "Claims 1 to 3" and insert --Claim 1--.

Claim 5, line 1, delete "Claims 1 to 4" and insert --Claim 4--.

Claims 6 and 8, line 1 of each, delete "Claims 1 to 5" and insert --Claim 1--.

Electrokinetic sample preparation

Biological macromolecules such as, for example, proteins and nucleic acids, but also small particles such as viruses and bacteria, are of great importance both in diagnosis and for medical research.

The established methods for characterizing these substances from biological matrices are in some cases very complicated. Thus, for example, for nucleic acid analyses the target nucleic acid is isolated and then amplified and subjected to a suitable analytical method. The isolation is time-consuming and difficult to automate. In order to obtain sufficient qualities of nucleic acid for the established analytical methods, the nucleic acid must be replicated by amplification in a further step. Only the polymerase chain reaction (PCR) has found relatively wide application for this hitherto.

It is possible with "electrokinetic sample preparation" to carry out the entire nucleic acid analytical procedure from the isolation to the analysis, also avoiding the additional amplification, with previously unknown rapidity and automation.

Purification and diagnosis of proteins

Since different proteins have individual physical properties, there is no universally applicable method for purification. Mainly chromatographic and electrophoretic methods, precipitations, ultrafiltration, ultracentrifugation and size exclusion chromatography (Doonan, S., *Methods Mol. Biol.*, **1996**, 59, Totowa, N.J., Humana, 1996, 405ff) are used. For diagnostic applications, immunological methods which detect the target protein via specific antibody recognition have become established.

Purification and diagnosis of nucleic acids

In order to isolate nucleic acids from biological material for diagnostic applications, various disruption methods and subsequent purification methods are necessary, depending on the nature of the material. It must moreover in turn be ensured that the nucleic acid to be isolated is not decomposed. RNA in particular may easily be degraded by ubiquitous RNAses, so that addition of inhibitors for these interfering enzymes is necessary (Walker, J.M., *Methods Mol. Biol.*, **1984**, 2, Clifton, N.J., Humana, 1984, 113ff). The methods customary at present are outlined below:

A simple case of obtaining nucleic acid is the isolation from a pure bacterial culture: in the case of E. coli, alkaline lysis liberates the nucleic acid; after centrifugation and neutralization, this crude product can be used directly for PCR mixtures (Rolfs, A. et al. PCR: Clinical Diagnosis and Research, Berlin, Springer, 1992).

However, as a rule, the sample material for medical diagnostic methods has a more heterogeneous structure; blood, urine, CSF, smear specimen, sputum, tissue samples, faeces, for example, require specific disruption methods which must in turn be modified depending on the objective (detection of bacteria, fungi, viruses or genomic nucleic acid from the carrier organism).

The following is a brief summary of the most usual methods for purifying these samples:

Phenol extraction of the nucleic acid with proteinase K treatment.

Retarding of the nucleic acid on a membrane filter: is used only for prepurified samples or samples with little contamination - such as urine - because of the problems of blockage.

Solid phases to which the nucleic acids can be bound make it possible to separate interfering and concomitant substances by washing steps. Examples thereof are: a) absorption on Glassmilk in sodium iodide buffer (Maiwald, M. et al., *BIOforum* **1994**, 17, 232-237). b) Attachment of the negatively charged nucleic acid to weakly basic polymers (EP 0707077 and US 5434270). c) Cellulose matrices for direct absorption of blood, after which all the treatment steps including nucleic acid liberation and purification take place. (Del Rio, S.A. et al. *Biotechniques*, **1996**, 20, 970-974). d) Silica microparticles, which may also be embedded in membranes, can likewise be employed for nucleic acid purification (WO 95/34569). Ion exchange membranes (US 832284) or chemically modified silica phases (EP 0648777) likewise belong to this group.

Electroelution apparatuses are also on the market (for example from Biometra) for macroscopic extraction of proteins, nucleic acids or viruses from gels (EP 380357).

In order to obtain sufficient quantities of nucleic acid, the isolation of the nucleic acids is normally followed by an amplification step (EP 229701).

Approaches to automation of nucleic acid analyses take the form of anion exchange chromatography and nucleic acid adsorption using robot pipettors (BioRobot from Qiagen). In a Japanese patent, the nucleic acid is immobilized in a capillary in order to be able to carry out the amplification therein (JP 7107962).

Purification and diagnosis of viruses

Viruses are normally isolated and concentrated by the following methods: ultracentrifugation, electroextraction, size exclusion separation, affinity chromatography and precipitation (Polson, Alfred, *Prep. Biochem.*, **1993**; 23, Dekker, New York, N.Y., 1993). For diagnostic purposes, either immunological methods for the protein coat or nucleic acid analysis methods after liberation of the viral nucleic acids are employed.

Purification and characterization of bacteria

Bacteria are normally isolated and cultivated by streaking methods on nutrient media. Available for isolation and characterization are immunological methods - for example by fluorescent labelling -, nucleic acid determination methods - after cell lysis.

All the methods, irrespective of the macromolecule, are time-consuming, include numerous dilution steps and frequently do not guarantee removal of interfering factors. The technical state of microchannel technology and of nucleic acid analysis without amplification will be briefly outlined below.

Microchannels

Capillary electrophoresis is a relatively young analytical separation technique (St. Claire R.L., *Anal. Chem.* **1996**, 68, 569R-586R). The principle is based on separation of analytes in an electrolyte-filled capillary by applying a high-voltage field between the ends of the capillary.

Capillary electrophoresis analytical methods have been employed for some years for analysing biological macromolecules such as proteins (WO 93/22665), nucleic acids (Heller, C., *J. Chromatogr. A.*, **1995**, 698, 19-31) and recently also viruses

(DE 4438833). Detection takes place either directly with UV or by means of fluorescence detection after labelling of the macromolecules (Pentoney, S.L., Jr., et al. *Handbook of Capillary Electrophoresis*, p. 147, Landers, J.P. (Ed.) Boca Raton, CRC Press, 1994). Almost all apparatus manufacturers provide analytical kits for nucleic acid analysis using CE. Since 1995 there has also been a completely automatic nucleic acid analyser based on CE, the ABI Prism 310 from Perkin-Elmer (Applied Biosystems). Injection of the nucleic acid into the capillary normally takes place electrokinetically by applying a voltage. However, the amount which can be loaded electrokinetically is limited since otherwise peak broadening and sample discrimination occurs (Butler, J.M., et al. *J. Chromatogr.*B, **1994**, 658, 271-280).

A marked increase in sensitivity was achieved by the introduction of laser-induced fluorescence detection into capillary electrophoresis (St. Claire R.L., *Anal. Chem.* **1996**, 68, 569R-586R), which has already been commercialized by Beckman. This makes it possible also to analyse intact viruses by CE (DE 4438833). Proteins can be detected by fluorescence only after specific modification.

Concentration in the microchannel

One disadvantage in principle of CE is the low injection volume which amounts to only a few nanolitres. A large number of attempts has been made to compensate for this disadvantage (St. Claire R.L., *Anal. Chem.* **1996**, 68, 569R-586R). These include isotachophoretic concentration and stacking, both of which lead to focusing of the sample constituents in the injection volume. A patent application was filed in 1993 (WO 93/05390) by Guzmann for a specific solid-phase adsorption in the capillary for sample concentration. Specific compounds in a sample are retained or allowed through by specific molecular interaction. A further development of these methods was made by Tomlinson et al. with membrane preconcentration capillary electrophoresis (Tomlinson, A.J., et al. *J. High Res. Chromatogr.* **1995**, 18, 381-383). Introduction of a reverse phase membrane into the capillary leads to lipophilic sample constituents being retained in a solid-phase extraction in the membrane, and then eluted with an organic solvent through the membrane and separated by capillary electrophoresis.

CE chips

Capillary Array Electrophoresis was developed by various research groups, mainly for nucleic acid sequencing, and a patent application has been filed in part (WO 96/04547). Fluorescent molecules underwent voltage-dependent control and analysis in microcapillary systems produced by photolithography.

Likewise based on chip technology, micromachines which make process control possible for solutions for synthetic or analytical purposes through a network of channels and electrical switches have been patented (WO 96/15450).

Amplification-free nucleic acid analyses

Based on the high sensitivities of fluorescence detection, a patent application has been filed for flow cytometry for single molecule detection (WO 90/14589).

Fluorescence correlation spectroscopy has likewise been employed for biological screening methods via single molecule detection (EP 731173). High throughput nucleic acid sequencing based on this technology is also being worked on (Harding, J.D., et al. *Trends in Biotechnology*, **1992**, 10, 55-57). These methods are based on the detection of a single fluorescent molecule in a very small volume element.

The intention of the present invention was to develop an automated method which permits macromolecules (nucleic acids, proteins, viruses and bacteria) from biological materials such as, for example, blood, blood plasma, serum, CSF, urine, tissue samples, plants, cells, cell supernatants etc. and preparations thereof to be isolated, concentrated and made available for analysis.

The central component of the sample preparation module is a microchannel which can be thermostatted and has an introduced membrane. This channel, which is filled with a conducting liquid, is in contact at both ends with exchangeable or permanently installed vessels. Applying a potential difference between the sample vessels makes electrophoretic mobilization of charged molecules possible. The possibility of applying a pressure difference additionally makes it possible to generate a laminar flow in the microchannel. Figs. 1-5 show a simplified diagrammatic representation.

A membrane (2) is introduced into a microchannel (1) and is suitable for retaining the required macromolecule. Part of the sample is injected into the channel by applying a pressure difference (6) and/or a voltage (5) to the ends of the microchannel (3, 4) (Fig. 1). Injection is continued until a sufficient quantity of the required macromolecule is present in the microchannel. The injection can be adjusted to suit the required target molecule by the combination of the parameters pH, membrane properties, characteristics of the microchannel, polarity of the voltage and direction of the pressure gradient, so that only the required macromolecule either enters the channel or is held by the membrane.

The microchannel has an internal diameter of 10-100 µm and a total length of 3-50 cm. The microchannel is made from an electrically nonconducting material such as polymer, ceramic, glass or quartz. All synthetic polymers are suitable in principle. The polymer must be inert towards the buffer solutions employed, and is ideally transparent for optical detection methods (for example polycarbonate, polyesteracrylate, polymethacrylate, polyurethane, polyacrylamide), but PTFE is also suitable. To obtain favourable surface properties, the channel can be coated on the inside with a polymer (for example polyacrylamide, silanol or polyvinyl alcohol).

Irrespective of the type of macromolecule investigated, it is possible to employ membranes which operate on the size exclusion principle (ultrafiltration membrane). The size exclusion range must be adapted to suit the molecular size of the macromolecule. The range of the membranes extends from Mw 3000 for small proteins or nucleotides, through size exclusion ranges in the lower nm range for large nucleic acids and viruses, up to 0.45 mm for bacteria and cells. The membranes comprise polymers with a microstructure, preferably polyethersulphone (PES), polyester, fabric-supported acrylic polymer, polytetrafluoroethylene (PTFE), polysulphone, polypropylene (PP), glass fibre, nylon or polycarbonate. It is additionally possible to employ ion exchange membranes and adsorption phases. However, the choice of these membranes depends on the nature of the macromolecule and is therefore treated individually.

After optimal change of the sample vessel (3) for a concentration buffer vessel (7), the injected macromolecule is concentrated in front of or in the membrane (2) by applying a pressure difference (6) and/or a voltage (5) (Fig. 2). The required macromolecule is then present in a volume of a few nanolitres.

After optimal change of the vessels (4, 7) for the reagent vessels (8, 9), the solutions present therein, or constituents thereof, can be brought into the microchannel (1) by applying a pressure difference (6) and/or a voltage (5) (Fig. 3). The conditions are chosen so that the target molecule remains concentrated during this. The target molecule can be enzymatically or chemically modified in this way, and/or be specifically recognized by hybridization or immunological recognition. The possibility of thermostatting the microchannel (1) and the possibility of changing the reagent vessels several times permit complex reactions and cyclic processes. Any derivatization reactions necessary for fluorescence, chemiluminescence or laser-induced fluorescence detection are also carried out in this modification step.

The required reaction temperatures are reached by thermostatting the microchannel (1). For this purpose, either liquid or air at an appropriate temperature is passed by the microchannel. The thickness of the microchannel wall is moreover chosen so that adequate removal of heat is ensured.

After optimal change of the reagent vessels (8, 9) for the buffer vessels (10, 11), the target molecule is mobilized by applying a pressure difference (6) and/or a voltage (5) (Fig. 4). The molecule can be determined analytically (13) directly in the microchannel (1) by optical detection methods (12) such as absorption or fluorescence. Detection methods analogous to those in CE are available (St. Claire R.L., *Anal. Chem.* **1996**, 68, 569R-586R).

For this purpose, the microchannel is made transparent, either completely or at one point, for optical radiation. The transmission of the excitation radiation and of the fluorescence radiation must moreover be ensured. The materials are preferably the nonconducting ones explained at the outset. The fluorescence radiation is measured in a defined angle of 0-180° perpendicular or in reflexion to the incident wavelength. Irradiation preferably takes place at 45° or 90°.

The highly concentrated analytical target is, however, also available for more extensive analyses (Fig. 5). Thus, the target molecule can be fractionated into the analysis vessel (14) or into or onto any other analytical target.

The analysis vessel (14) contains 1-1000 ml of a buffer suitable for the further analysis. This is, for example, PBS buffer, tris/borate or a tris/glycine buffer. The analysis vessel (14) may be a flat analytical target, for example a mass spectrometry

sample carrier. Electrical contact is made either directly through the conducting analytical target or by wetting the surface between electrode and microchannel with an electrically conducting liquid.

If the channel is additionally branched, the concentrated target molecule can be brought, by switching over the pressure or voltage, into other channels for further analyses and is therefore directly compatible with CE chip technology (WO 96/04547).

Fractionated macromolecules can be further analysed by all conceivable methods. The highly concentrated macromolecule is eluted in less than one microlitre and can be loaded directly into suitable liquid matrices or else onto solid sample carriers.

A diagrammatic representation of the parallel design is to be found in Fig. 6. The microchannels (1) are produced from nonconducting materials such as polymer, ceramic, glass or quartz (15) and coated where appropriate. The channel blocks are joined together with an intermediate membrane layer (2) so that the channels meet at the membrane. The arrangement of the channels depends on the sample format. The following additional devices are not depicted. To remove Joule's heat, where appropriate additional thermostatting elements are introduced. The channels are tapered at the ends so that they can be introduced into the respective vessels or are tightly connected to permanently installed vessels. For electrical contact, electrodes are attached either to the channel ends or inside the vessels and are supplied from a high voltage source.

For the thermostatting, for example, channels which lie perpendicular to the direction and between the planes of the microchannels are placed in the analysis block. Liquid or air at an appropriate temperature can be pumped through these channels.

For distinctly faster concentration of the samples, a parallel capillary arrangement has been developed. Diagrammatic representation of this module is to be found in Fig. 7. Beside the microchannel (1) there is the distinctly wider channel (16). The height of the channel corresponds to the dimension of the microchannel (10-100 µm) so that Joule's heat can still be removed efficiently. However, the width of the channel (100 µm to 10 mm) allows flow rates up to 10^3 higher than in the microchannel (1). The membrane is clamped between the module blocks (15). The entire module is thus 3 to 10 cm long, 1 to 50 mm wide and 0.1 to 50 mm thick. The channel ends are

in turn connected to either exchangeable or permanently installed sample vessels. It is also possible by parallel arrangement to achieve a design analogous to that in Fig. 6. The macromolecules are concentrated in the channel (16) and then transferred through the transfer channel (17) into the microchannel and further processed in analogy to the methods from Figs. 1-6. Schematic enrichment of macromolecules using this rapid concentration is described below for the example of nucleic acids.

The enrichment of nucleic acid from salt-containing solution takes place by applying a voltage to the shallow channel (16) (Fig. 8a). Besides the excess of small anions (small black beads), the nucleic acid from the sample is also injected. The anionic molecules migrate through the membrane (2) and are thus removed from the nucleic acid. The voltage is maintained until all nucleic acids are immobilized on the membrane surface (Fig. 8b). If a voltage is now applied as indicated between the shallow channel (16) and the microchannel (1), then the purified and concentrated nucleic acid migrates from the large membrane surface of the shallow channel (16) to the membrane of the microchannel (Fig. 8c) and is subsequently available for further treatment and analysis (Figs. 3-6). It is also possible during this transfer step simultaneously to carry out modification reactions on the concentrated macromolecule.

1. Description of nucleic acid concentration

1.1 Nucleic acid is liberated by a suitable, established method (lysis, hydrolysis, ultrasound etc.) from the sample to be investigated and is mixed with a suitable acidic extraction buffer. The buffer must be designed so that non-nucleotide constituents of the solution do not carry an excess anionic charge. Below a pH of about 5, proteins no longer show an excess negative charge. It is possible to employ inorganic acids such as hydrochloric acid, phosphoric acid or sulphuric acid as well as organic phosphoric acid derivatives or sulphonic acids. However, a polymer-bound, acid ion exchanger (for example polystyrenesulphonic acid) is preferably employed. The acidic pH is achieved by using the ion exchanger, without introducing additional anions into the solution. This favours electrokinetic nucleic acid extraction.

1.2 The nucleic acid is extracted from this acid-buffered solution by electrophoresis. For this purpose, an electrode is introduced into the

solution and brought to a cationic potential. The electrode in the buffer vessel (4) (Fig. 1) is brought to an anodic potential, and the microchannel (1) is filled with an electrically conducting liquid. The composition of the electrolyte depends on the nature of the immobilization technique. It is preferably an aqueous borate-, phosphate- or citrate-based buffer. Glycine is also a suitable buffer ion. The buffer concentration is between 10 and 100 mmol/l. The pH is between 2.5 and 8.5. For example sodium citrate (20 mmol/l, pH 5.0) or tris/borate (100 mmol/l, pH 8.5). A modifier, preferably a chaotropic agent such as, for example, urea in molar concentration, is optionally added. The loading can be followed by UV or fluorescence detection - after previous derivatization - in the microchannel (1).

1.3 The extracted nucleic acid is immobilized and concentrated in the channel with the aid of the introduced membrane while maintaining the voltage (Fig. 2). In addition to the size exclusion membranes, also suitable for nucleic acids are the soft anion exchangers, for example based on amines. Alkylamine-, imidazole- or pyrrolidone-substituted polymers are preferred. The nucleic acids can also be retarded by adsorption on membranes. The membranes contain, for example, nanoparticles, preferably silica-based or metal oxide pigments. Specific nucleic acid strands are immobilized by solid-phase hybridization with immobilized oligonucleotides.

1.4 The nucleic acid which has been concentrated to a few nanolitres can be modified and analysed in a variety of ways (Fig. 3). The open channel system allows reagents to be introduced and removed by pressure or voltage. The polarity of the voltage is maintained as in the extraction and focusing. Combined with a suitable temperature control, enzymatic cleavages, Sanger sequencings, gene probe hybridization, but also PCR reactions, are possible in the microsystem.

For example, the nucleic acid can be labelled with intercalating dyes. These are preferably fluorescent derivatives such as ethidium bromide, acridine orange, or dimers thereof, such as 1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium tetraiodide (YOYO). The choice of

the dye depends primarily on the chosen detection unit. YOYO is, for example, ideal for fluorescence detection after excitation with an argon laser, whereas the corresponding YOPRO dimer is ideally suited for the infrared laser. These dyes are also distinguished in that scarcely any background fluorescence occurs, because these dyes fluoresce only in the intercalated state. The positively charged YOYO can be introduced, for example, electrokinetically from the reaction vessel (9) while switching in the focusing voltage.

Distinctly greater specificity can be achieved, for example, by a fluorescence-labelled gene probe. The gene probe consists of a nucleotide sequence which is complementary to the target nucleic acid and which carries one or more fluorescent dyes. The choice of the dye depends primarily on the chosen detection unit. Fluorescein isothiocyanate or reactive coumarin derivatives are preferably employed for fluorescence detection after excitation with an argon laser.

Enzyme-catalysed reactions such as restriction enzyme digestion, PCR reaction and Sanger sequencing are carried out by transporting the required enzymes and substrates needed to the nucleic acid in the microchannel.

1.5 The concentrated nucleic acid can be eluted in a few nanolitres in a suitable buffer by applying pressure and/or voltage and is available for further analyses.

For this purpose, the polarity of the voltage is reversed so that the anode is now in the analysis vessel (14) (Fig. 5). It is preferred for the microchannel and buffer vessel (11) to be filled previously with an aqueous buffer of the abovementioned composition. The subsequent analyses which can be carried out are PCR reaction, CE separations, DNA sequencings, hybridization reactions, mass spectrometric analysis or molecular biological methods.

1.6 The nucleic acid can be analysed by electrophoresis within the microsystem (Fig. 4). In this case, as for the elution, the buffer vessel

(10) is brought to an anodic potential. Nucleic acid fragments can be separated as a function of size in a suitable sieve medium.

The buffer vessels (10, 11) and the microchannel (1) are for this purpose filled with a polymer-containing buffer solution. This preferably comprises linear soluble polymers, for example acrylamide, polyvinyl alcohol, cellulose (modified and unmodified), dextran or agarose. The remaining composition of the buffer corresponds to the general composition.

1.7 Direct fluorescence detection of the nucleic acid in the microsystem is possible by using fluorescence-labelled probes, fluorescence-labelled terminators in the Sanger sequencing or intercalating dyes.

2. Viruses

2.1 The virus-containing sample is brought to a pH such that the virus to be investigated, or the viruses to be investigated, carries an excess negative charge. It is possible, if necessary, beforehand to carry out nuclease digestions or virus modifications.

Suitable buffers have already been described in the general method. The pH of the buffer must be distinctly higher than the pK of the virus. Acidic buffers such as sodium citrate are therefore dispensed with, and likewise modification reagents are not used. Nuclease digestions are preferably carried out by adding RNAses or DNAses. An outstandingly suitable example is benzonase. Modification reactions can be carried out in the form of staining reactions with intercalating dyes (cf. 1.4) or by incubation with fluorescence-labelled antibodies. The choice of the dye depends in both cases on the chosen mode of detection.

2.2 The negatively charged viruses are extracted from this buffered solution by electrophoresis. For this purpose, an electrode is introduced into the solution and brought to a cationic potential. The electrode in the buffer vessel (4) (Fig. 1) is brought to an anodic potential and the microchannel (1) is filled with an electrically conducting liquid. The composition of the electrolyte corresponds to the general composition of the buffer. The

loading can be followed by UV or fluorescence. During the injection, a pressure difference can additionally be applied between the buffer vessels (3 and 4).

2.3 The extracted viruses are immobilized and concentrated in the channel with the aid of the introduced membrane (Fig. 2). The membrane operates on the size exclusion principle, with the pore size being between 10 and 200 nm depending on the virus.

2.4 The viruses which have been concentrated to a few nanolitres can be modified and analysed in a variety of ways (Fig. 3). The open channel system permits reagents to be introduced and removed by means of pressure or voltage. Combined with a suitable temperature control, all derivatization methods for proteins and nucleic acids are possible in the microsystem (cf. 1.4 and 3.4). The viruses can also be lysed on the membrane, and the nucleic acids and/or the proteins can subsequently be analysed (cf. 1.4-1.7 and 3.4-3.7).

In the case of virus lysis, the size exclusion membrane must have dimensions such that the target proteins or nucleic acids are likewise retarded. Every lysis protocol is suitable in principle. It is preferred to use denaturing conditions such as extreme pH values, chaotropic reagents or detergents. Examples are dilute sodium hydroxide solution, guanidinium hydrochloride or sodium dodecyl sulphate (SDS). The lipoprotein membrane can be removed from enveloped viruses by using nonionic detergents (for example NP-40).

2.5 The concentrated viruses can be eluted in a few nanolitres in a suitable buffer by applying pressure and/or voltage and are available for further analyses.

For this purpose, the polarity of the voltage is reversed so that the anode is now in the analysis vessel (14) (Fig. 5). It is preferred for the microchannel (1) and the buffer vessel (11) to be filled previously with an aqueous buffer of the abovementioned composition. After fractionation into a buffer-filled analysis vessel (14) it is possible to carry out, for example, pathogenicity assays or CE separations with the viruses.

After fractionation onto a flat analytical target (14) it is possible for the viruses for example to be examined by direct electron microscopy.

2.6 The viruses can be analysed by electrophoresis within the microsystem. In this case, as for the elution, the buffer vessel (10) is brought to an anodic potential (Fig. 4).

2.7 The viruses can be identified by fluorescence spectroscopy by use of fluorescence-labelling methods for proteins or nucleic acids (cf. 1.4 and 3.4).

3. Proteins

3.1 The protein-containing sample is brought to a pH which is at least one log stage close to the pK of the protein. If the solubility properties of the protein permit, the pH is set below the pK of the protein so that the protein is in the positively charged state. This case will be discussed through below. For negatively charged proteins, the voltage relationships are correspondingly inverted. Suitable buffers correspond to the general conditions. Alkaline-buffered phosphate and citrate buffers are preferably used, for example sodium citrate, 20 mmol/l, pH 2.5.

3.2 The positively charged proteins are extracted from this buffered solution by electrophoresis. For this purpose, an electrode is introduced into the solution and brought to an anionic potential. The electrode in the buffer vessel (4) (Fig. 1) is brought to a cathodic potential and the microchannel (1) is filled with the buffer. The composition of the electrolyte depends on the type of immobilization technique and corresponds to the general conditions. A modifier, preferably an organic solvent such as, for example, methanol between 5 and 30%, is optionally added. The loading can be followed by UV or fluorescence detection - after previous derivatization - in the microchannel (1).

3.3 The extracted proteins are immobilized and concentrated in the channel with the aid of the introduced membrane (Fig. 2). Besides the size exclusion membrane, also suitable for proteins are ion exchange membranes. Soft anion exchangers preferably used for negatively

charged proteins are DEAE phases. The strong anion exchangers mainly used are quaternary ammonium phases. In the case of the cationic proteins discussed here, suitable soft exchangers are mainly carboxylic acid phases and strong exchangers are sulphonic acid phases. For specific proteins, the membranes can be coated with appropriate antibodies and thus be affinity-enriched.

3.4 The proteins which have been concentrated to a few nanolitres can be modified and analysed in a variety of ways (Fig. 3). The open channel system permits reagents to be introduced and removed by means of pressure or voltage. The polarity of the voltage is maintained during the extraction and focusing. Combined with a suitable temperature control, enzymatic cleavages, complexations, chemical derivatizations or antibody bindings are possible in the microsystem.

For example, the protein can be reacted with reactive dyes. These are preferably amine-specific dyes such as, for example, fluorescein isothiocyanate (FITC). The choice of the dye depends primarily on the chosen detection unit. FITC is, for example, ideal for fluorescence detection after excitation with an argon laser.

Distinctly greater specificity can be achieved, for example, by fluorescence-labelled antibodies. In the subsequent separation, the protein-antibody complex is then determined by fluorescence.

3.5 The concentrated proteins can be eluted in a few nanolitres in a suitable buffer by applying pressure and/or voltage and are available for further analyses.

For this purpose, the polarity of the voltage is reversed so that the cathode is now in the analysis vessel (14) (Fig. 5). It is preferred for the microchannel and the buffer vessel (11) to be filled previously with an aqueous buffer of the abovementioned composition. Subsequent analyses which can be carried out are CE separations, mass spectrometry analyses, enzyme assays, binding studies or immunological methods.

3.6 The proteins can be analysed by electrophoresis within the microsystem (Fig. 4).

3.7 The proteins can also be identified by fluorescence spectroscopy by using fluorescence-labelled antibodies, fluorescent enzyme substrates, fluorescent binding partners or derivatization reagents.

4. Bacteria

4.1 The bacteria-containing sample is brought to a pH such that the bacterium to be investigated carries an excessive negative charge. If necessary, nuclease digestions or protein modifications can be carried out beforehand.

The suitable buffers have already been described in the general method. The pH of the buffer must be distinctly higher than the pK of the bacterium. Acidic buffers such as sodium citrate are therefore dispensed with, and modification reagents are likewise not used. Nuclease digestions are preferably carried out by adding RNAses or DNAses. An outstandingly suitable example is benzonase. Modification reactions can be carried out in the form of staining reactions with intercalating dyes (cf. 1.4) or by incubation with fluorescence-labelled antibodies. The choice of the dye depends in both cases on the chosen mode of detection.

4.2 The negatively charged bacteria are extracted from this buffered solution by electrophoresis. For this purpose, an electrode is introduced into the solution and brought to a cationic potential. The electrode in the buffer vessel (4) (Fig. 1) is brought to an anodic potential and the microchannel (1) is filled with an electrically conducting liquid. The composition of the electrolyte depends on the nature of the immobilization technique. The loading can be followed by UV or fluorescence.

4.3 The extracted bacteria are immobilized and concentrated in the channel with the aid of the introduced membrane (Fig. 2). The membrane operates on the size exclusion principle (ultrafiltration membrane) or the ion exchange principle (anion exchanger). The membranes preferably used for the sterile filtration have an exclusion size of 0.1 - 0.45 µm.

However, it is also possible to employ the same anion exchangers as for the proteins (cf. 3.3).

4.4 The bacteria which have been concentrated to a few nanolitres can, for example, be lyophilized on the membrane and then the proteins or nucleic acids can be modified and analysed in a variety of ways (Fig. 3). The open channel system permits reagents to be introduced and removed by means of pressure or voltage. Combined with a suitable temperature control, all derivatization methods for proteins and nucleic acids are possible in the microsystem (cf. 1.4-1.7 and 3.4-3.7).
The size exclusion membrane must in the case of bacterial lysis have dimensions such that the target proteins, or nucleic acids, are likewise retarded. Every lysis protocol is suitable in principle. Denaturing conditions are preferably used, such as extreme pH values, chaotropic reagents or detergents. Examples are dilute sodium hydroxide solution, guanidinium hydrochloride, urea or sodium dodecyl sulphate (SDS).

4.5 The concentrated bacteria can be eluted in a few nanolitres in a suitable buffer by applying pressure and/or voltage and are available for further analyses.

For this purpose, the polarity of the voltage is reversed so that the anode is now in the analysis vessel (14) (Fig. 5). It is preferred for the microchannel (1) and the buffer vessel (11) to be filled previously with an aqueous buffer of the abovementioned composition. After fractionation into a buffer-filled analysis vessel (14) it is possible to carry out, for example, pathogenicity assays or electrophysiological experiments with the bacteria. After fractionation onto a flat analytical target (14) it is possible for the bacteria to be examined, for example, by direct light or electron microscopy, or to be identified, for example, microbiologically via plaques on an agar plate.

4.6 The bacteria can be analysed by electrophoresis within the microsystem (Fig. 4).

4.7 The bacteria can also be identified by fluorescence spectroscopy by using fluorescence-labelled antibodies or fluorescent binding partners.

A precondition for all conventional methods of macromolecule isolation is processing of the complete sample volume by the extraction medium. However, manipulation of large-volume samples prevents miniaturization, which is in turn absolutely necessary in order to increase the speed of analysis and the sensitivity. The combination of direct electrophoretic extraction with an immobilization membrane on the microscale makes it possible for the first time to combine large sample volumes directly with a nanoanalytic technique.

Compared with established methods, the method is distinguished in particular by the simplified isolation and exceptional enrichment rates.

If the sample is eluted from the microchannel by electrophoresis or using pressure, it is subsequently possible to carry out further nanoanalytical methods. After dilution, the isolated sample is also available for conventional macroscopic analytical methods. The method then represents a very efficient sample preparation module for these techniques.

It is possible by concentrating nucleic acids in many cases to avoid additional amplification steps. The method can thus replace PCR, for example.

The method can also be employed as disruption method for viruses, bacteria and other cells. For example, bacterial material is isolated and then the bacterium is disrupted in the microchannel, and the liberated nucleic acid is derivatized and analysed.

The method can be employed advantageously for direct nucleic acid sequencing for diagnosis and research. In the case of human DNA this makes it possible to analyse for inherited genetic defects caused by deletions, mutations or translocations. Possible areas of use which may be mentioned here are: cystic fibrosis, Down's syndrome, sickle-cell anaemia, Huntington's chorea, haemophilia A and B. Another application of this nucleic acid analysis is in tumour diagnosis and the general identification of genetic predispositions for certain diseases. In this connection, analysis of tumour suppressor genes and oncogenes is of particular interest.

Another use is in combination with nucleic acid amplification methods (such as, for example, PCR).

The method can also be employed for direct gene probe analysis of drug-resistant organisms or for subclassification.

As quality assurance method, the invention also makes it possible to monitor genetically engineered products for which freedom from nucleic acids must be ensured.

Investigation of intact viruses, bacteria or their nucleic acid or their proteins can be employed for infection diagnosis. Nucleic acids or proteins from fungi or parasites can also be analysed for these purposes. In the case of the viruses, the most important viral representatives which may be mentioned here by way of example are HIV 1 and 2, HTLV, HSV, CMV, HPV, Hepatitis A, B, C, D, E, F, G, VZV, rotaviruses, EBV and adenoviruses. The most important bacterial representatives include chlamydias, mycobacteria, shigella, campylobacter, salmonellas, neisserias, staphylococci, streptococci, pneumococci. In the case of fungi, the most important pathogens include candida, aspergillus and cryptococcus.

Another area of use is to be regarded as the safety monitoring of biological samples. An example of the importance of this is in the testing of donated blood and all products manufactured from blood. The areas of use substantially correspond to those of infection diagnosis.

This method permits for the first time the direct high-sensitivity detection of intact viruses. It is moreover possible to measure directly any viruses, including unknown ones. This has enormous significance both for infection diagnosis and for the safety of products from biological materials, because it is possible with all other methods to detect only specific viruses individually.

The proteins obtained by electrokinetic sample preparation are, because of their enrichment and purification, more readily amenable to subsequent immunodiagnostic analysis. In this connection, various proteins such as, for example, transferrin, fibrinogen, β-2 microglobulin, hCG or tumour markers (AFP, CEA, CA 15-3, CA 19-9) have great diagnostic relevance in human diagnosis.

Examples

Electrokinetic injection of nucleic acid

To check the electrokinetic injection of nucleic acid, model DNA was injected into a microchannel by applying voltage and was measured by UV. The experiment was intended to show that it is possible to extract nucleic acid electrokinetically.

Serial dilutions of pBr DNA (Boehringer Mannheim) from 250 to 1.25 mg/l were prepared and placed in the sample vessel. Injection was followed by electrophoretic separation. The measurement conditions are shown in Table 1.

A plot of the peak areas against the DNA concentration shows a saturation of the increase in peak areas at high DNA concentrations. The injected amount of DNA above 100 mg/l is limited by the electric current and not by the DNA concentration. It was thus possible to show that nucleic acid can be concentrated electrokinetically from a solution over a wide concentration range.

Table 1: Measurement parameters for the electrokinetic nucleic acid injection.

Voltage:	-20 kV
Buffer:	Sodium citrate (20 mmol/l, pH 5.0, Fluka)
Capillary:	PVA-coated quartz capillary, 50 µm internal diameter, 64.5 cm length, 56 cm to the detector (Hewlett-Packard, Waldbronn)
Capillary electrophoresis apparatus:	^{3H}HPCE (Hewlett-Packard, Waldbronn)
Temperature:	25°C
Detection:	DAD 190-600 nm, λ 260 ± 8 nm
Injection:	Electrokinetically (20 sec × - 10 kV)
Capillary flushing before the injection:	1. Water (1 min, 5×10^4 Pa) 2. Buffer (3 min, 5×10^4 Pa)

Size exclusion membrane recovery rate

To check the retardation of nucleic acid on a size exclusion membrane, pBr DNA was injected electrokinetically into the microchannel and then mobilized electrophoretically in the channel to the anode. A UV detector was located between the injection end of the channel and the size exclusion membrane present in the channel (cf. Fig. 4). The pBr DNA (250 mg/l) was electrophoresed past the detector, and the electrophoresis was continued until the DNA would have left the channel without the membrane. The polarity of the voltage was then reversed and thus the retarded DNA was again moved past the detector. The measurement parameters are shown in Table 2.

Table 2: Measurement parameters for the electrophoretic nucleic acid recovery from a size exclusion membrane.

Voltage:	-10 kV for 10 min, then +10 kV for 10 min
Buffer:	Tris/borate (100 mmol/l, pH 8.5)
Capillary:	Coated quartz capillary, 75 µm internal diameter, 34 cm length, 8.5 cm to the detector (Biorad, Munich)
Membrane:	After 20 cm, the capillary was divided and, after introduction of the membrane (memfil PCTE 10 nm from Membrapure), reconnected with a Teflon shrinkable tube.
Capillary electrophoresis apparatus:	^{3H}HPCE (Hewlett-Packard, Waldbronn)
Temperature:	25°C
Detection:	DAD 190-600 nm, λ 260 ± 8 nm
Injection:	Electrokinetically (10 sec × - 10 kV)
Capillary flushing before the injection:	1. Water (1 min, 5×10^4 Pa) 2. Buffer (3 min, 5×10^4 Pa)

The electrokinetically injected DNA migrated after 1.7 min through the UV detector. Without a size exclusion membrane, the DNA would leave the channel after 7 min. The electrophoresis was, however, continued for a total of 10 min, and then the polarity of the voltage was immediately reversed. The retarded DNA in the capillary migrated back through the UV detector again. After 12.05 min it was possible to detect a signal which corresponded exactly to the peak area of the injected DNA. The

analogous experiment with 3-nitrobenzenesulphonic acid showed, as expected, only the injection peak and thus no retardation on the size exclusion membrane.

In another experiment, three successive injections of nucleic acid were carried out and analysed under identical conditions. The electrophoretogram unambiguously showed the concentration of the three nucleic acid injections in one signal. The peak areas of the three single injections corresponded exactly to the peak area of the retarded DNA. It was thus shown that macromolecules can be electrophoretically immobilized on the membrane in the microchannel and be quantitatively remobilized.

Nucleic acid extraction

For the nucleic acid concentration it is necessary to transfer the available amount of nucleic acid as quantitatively as possible from a particular solution into the microchannel. If the nucleic acid is completely injected, only very little nucleic acid ought now to be extractable from the same sample vessel in a second injection.

In order to minimize the amount of DNA, the DNA was stained with the intercalating dye YOYO (Molecular Probes) and detected by laser-induced fluorescence detection (LIF). For this purpose, 1-4 µl of the pBr DNA solution (1 mg/l) were added to YOYO (0.4 mmol/l, in 76 µl of TBE buffer) and incubated at RT for at least 30 min before the measurement. The measurement parameters are shown in Table 3.

Table 3: Measurement parameters for the electrophoretic nucleic acid extraction.

Voltage:	-10 kV for 10 min, then +10 kV for 10 min
Buffer:	Tris/borate (100 mmol/l, pH 8.5)
Capillary:	Coated quartz capillary, 75 µm internal diameter, 57 cm length, 50 cm to the detector (Biorad, Munich)
Capillary electrophoresis apparatus:	PACE 5510 (Beckman, Munich)
Temperature:	25°C
Detection:	LIF (Argon) EX 488, EM 520 nm, Gain 100
Injection:	2 × electrokinetically (60 min × - 10 kV) from 50 µl
Capillary flushing before the injection:	1. Water (1 min, 5×10^4 Pa) 2. Buffer (3 min, 5×10^4 Pa)

The detection profiles revealed a marked increase in the fluorescence after about 10 min, which rapidly reached a plateau. After about 30 min, the fluorescence declined again, associated with a gradual increase in the current in the channel. The level of the plateau correlated with the amount of DNA. No significant fluorescence was injectable from any sample in the second injection. The data demonstrate the complete extraction of the pBr DNA in the 1st injection. We conclude from the change in the fluorescence that all the nucleic acid was completely injected after only 30 min.

These data show unambiguously that all the nucleic acid in a sample volume can be injected electrokinetically into a microchannel. In combination with the size exclusion membrane, it ought to be possible to concentrate this nucleic acid into a few nanolitres and thus make it detectable.

Nucleic acid concentration

Nucleic acid was injected into the microchannel with incorporated membrane using the measurement conditions in Tab. 2. For this purpose, pBr DNA (2.5 mg/l, 50 µl) was injected for 25 min and then focused with the separation buffer (Fig. 2). The DNA was not directly detectable at this low concentration. After 10 min, the polarity of the voltage was reversed and the concentrated DNA migrated after 12 min as intense peak back through the detector. Virtually all the DNA in the 50 µl sample

(0.1 mg) was concentrated into less than 50 nl (1 cm in the capillary). The enrichment factor was thus a factor of 1000.

Derivatization on the membrane

To check the derivatization of nucleic acid on a size exclusion membrane, pBr DNA was electrokinetically injected and immobilized on the membrane as described above (Tab. 2). However, the voltage was not reversed immediately after the immobilization, but the buffer vessel located on the other side of the membrane from the DNA was replaced by a buffer solution containing cationic intercalating dye (YOYO, 0.4 mmol/l, Molecular Probes). The voltage was maintained for a further 20 minutes, during which the dye migrated through the microchannel and through the membrane. The DNA was thus incubated with YOYO on the membrane. Then electrophoresis was carried out without dye for a further 10 min in order to remove remaining YOYO from the capillary. The polarity of the voltage was then reversed, and the DNA retarded and stained in this way was again moved past the detector.

The electrokinetically injected DNA migrated through the UV detector after 2 min. Two minutes after the reversal of polarity it was possible to detect a signal with a larger peak area, which corresponded to the stained DNA. The UV-VIS spectrum of the injected DNA generated by diode array detection (DAD) in the microchannel showed the typical UV spectrum with 2 absorption maxima at 200 and 260 nm. After incubation with YOYO on the membrane, the DNA additionally showed an absorption maximum at 490 nm. This corresponds exactly to the absorption maximum of DNA intercalated with YOYO. It was thus possible to demonstrate that macromolecules can be derivatized in the microchannel.

Coupling with electron microscopy

Coupling with electron microscopy (EM) is intended to demonstrate further analysis of prepared macromolecules.

Herpes simplex viruses (Type 2) were stained with YOYO (Molecular Probes) and detected by laser-induced fluorescence detection (LIF). For this purpose, 4 µl of HSV-2 solution (5×10^5 viruses/ml) were added to YOYO (0.4 mmol/l, in 76 µl of TBE buffer) and incubated at RT for at least 30 min before the measurement. The measurement parameters are shown in Table 3.

The few injected, intercalated viruses (10 - 20) were detected as individual signals. To identify the signals, the same sample was analysed under comparable electrophoresis conditions in a Prince CE (Lauerlabs) with a nanofraction collector (Probot, BAI Instruments). The end of the microchannel was in this case fractionated under time control onto different electron microscopy carriers and, after negative contrasting with uranyl acetate, examined by EM.

It was possible unambiguously to detect HSV particles in the expected fractions. This showed for the first time with viruses as examples that macromolecules are available after electrophoretic separation as intact particles for further analyses.

Keys to the figures

Fig. 1: Diagrammatic representation of the purification apparatus. A microchannel (1) with incorporated membrane (2) connects 2 buffer vessels (3, 4). These buffer vessels can be brought to different voltages (5) via incorporated electrodes. It is also possible to apply various pressures via a pressure control device (6). The sample to be investigated is in (3).

Fig. 2: Diagrammatic representation of the concentration. The macromolecule to be investigated was injected electrokinetically into the microchannel (1) with incorporated membrane (2). The sample vessel has already been replaced by the concentration buffer (7). The macromolecules migrate as far as the membrane and are retained there.

Fig. 3: Diagrammatic representation of sample modification. The derivatization reagents are present in the reaction vessels (8, 9) and are brought to the concentrated macromolecules either electrokinetically and/or with the aid of pressure.

Fig. 4: Diagrammatic representation of an on-line analytical method. The concentrated and modified macromolecule is mobilized electrokinetically in the microchannel (1) past a detector window (12) so that the spectroscopic properties can be analysed and evaluated (13).

Fig. 5: Diagrammatic representation of the fractionation of the purified macromolecule. The concentrated sample is collected in the sample vessel or on the analytical target (14) and is available for further analyses.

Fig. 6: Diagrammatic representation of the high-throughput purification apparatus. A plurality of microchannels is arranged so that they are compatible with the sample format (for example microtitre plate). The membrane (2) is incorporated over the entire format and is connected to a second microchannel array (15). The procedure with these multiple arrangements corresponds to Figs. 1-5.

Fig. 7: Diagrammatic representation of the enrichment apparatus for rapid concentrations. Beside the microchannel (1) there is a shallow channel (16) which is connected to the microchannel (1) via the transfer channel (17). The shallow channel allows for much higher flow rates and thus larger enrichment factors. Details in the text.

Fig. 8: Diagrammatic representation of the rapid enrichment from salt-containing solution viewed from above with nucleic acids as example. The enrichment takes place by applying a voltage (6) to the shallow channel (16) (Fig. 8a). The voltage is maintained until all nucleic acids are immobilized on the membrane surface (2) (Fig. 8b). If a voltage (6) is now applied between the shallow channel (16) and the microchannel (1), as indicated, then the purified and concentrated nucleic acid migrates from the large membrane surface of the shallow channel (16) to the membrane of the microchannel (1) (Fig. 8c) and is then available for the analogous methods (Figs. 3-6). Details in the text.

Patent claims

1. Method for the isolation and concentration of macromolecules, characterized in that the macromolecules are electrokinetically collected on a membrane in a microchannel and are then analysed.

2. Method according to Claim 1, characterized in that the nucleic acids, viruses, proteins, bacteria or fungi are electrokinetically collected on a membrane in a microchannel and are then analysed.

3. Method according to Claims 1 and 2, where the sample is derivatized on the membrane.

4. Method according to Claims 1 to 3 as sample preparation for further analytical methods.

5. Method according to Claims 1 to 4 as sample preparation for MS, gel electrophoresis, PCR, TEM, nucleic acid sequencing, immunodiagnosis and hybridizations.

6. Device for carrying out the method according to Claims 1 to 5 in the form of a chip module with embedded membrane, with 1-400 capillaries being arranged side by side.

7. Device according to Claim 6, comprising a membrane of polyethersulphone (PES), polyester, fabric-supported acrylic polymer, polytetrafluoroethylene (PTFE), polysulphone, polypropylene (PP), glass fibre, nylon or polycarbonate.

8. Device for carrying out the method according to Claims 1 to 5 in the form of a shallow channel for analysing salt-containing samples.

9. Use of the device according to Claims 6 to 8 for the enrichment and the analysis of macromolecules.

10. Use of the device according to Claims 6 to 8 in the quality control of biological products.

11. Use of the device according to Claims 6 to 8 for direct infection diagnosis.

12. Use of the device according to Claims 6 to 8 for amplification-free nucleic acid analysis.

Electrokinetic sample preparation

Abstract

The present invention relates to a method which permits macromolecules (nucleic acids, proteins, viruses and bacteria) from biological materials such as blood, serum, CSF, urine, plants, cells, cell supernatants etc. and preparations thereof to be isolated, concentrated and made available for analysis. The macromolecules are initially concentrated electrokinetically on a membrane in a shallow channel. The concentrated macromolecules can be transferred electrokinetically via a transfer channel into an analytical microchannel for further processing.

Fig. 1

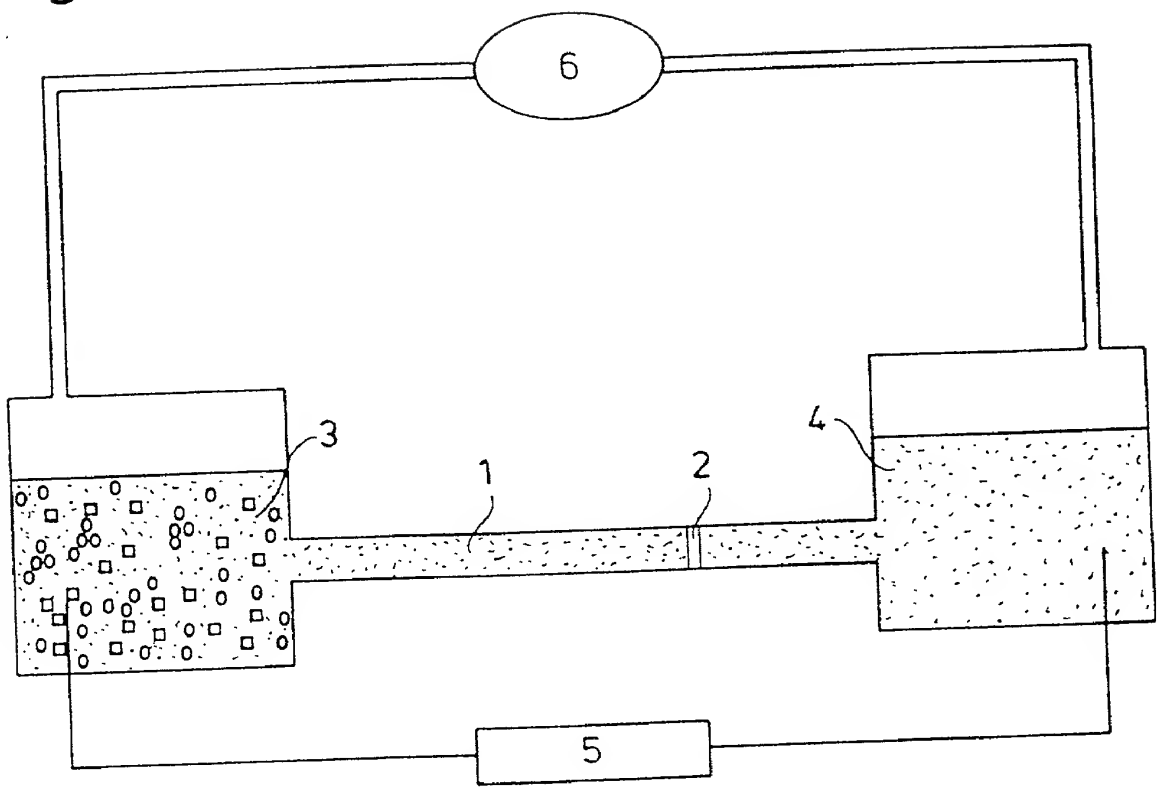


Fig. 2

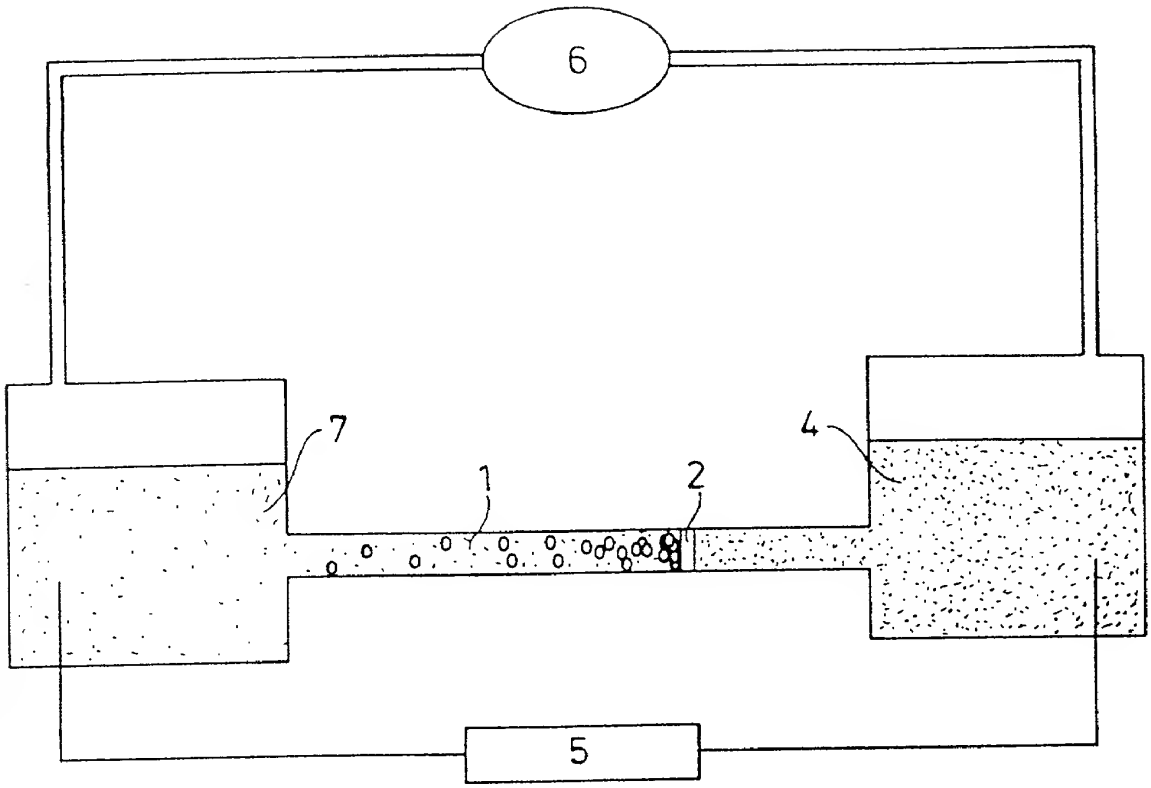


Fig. 3

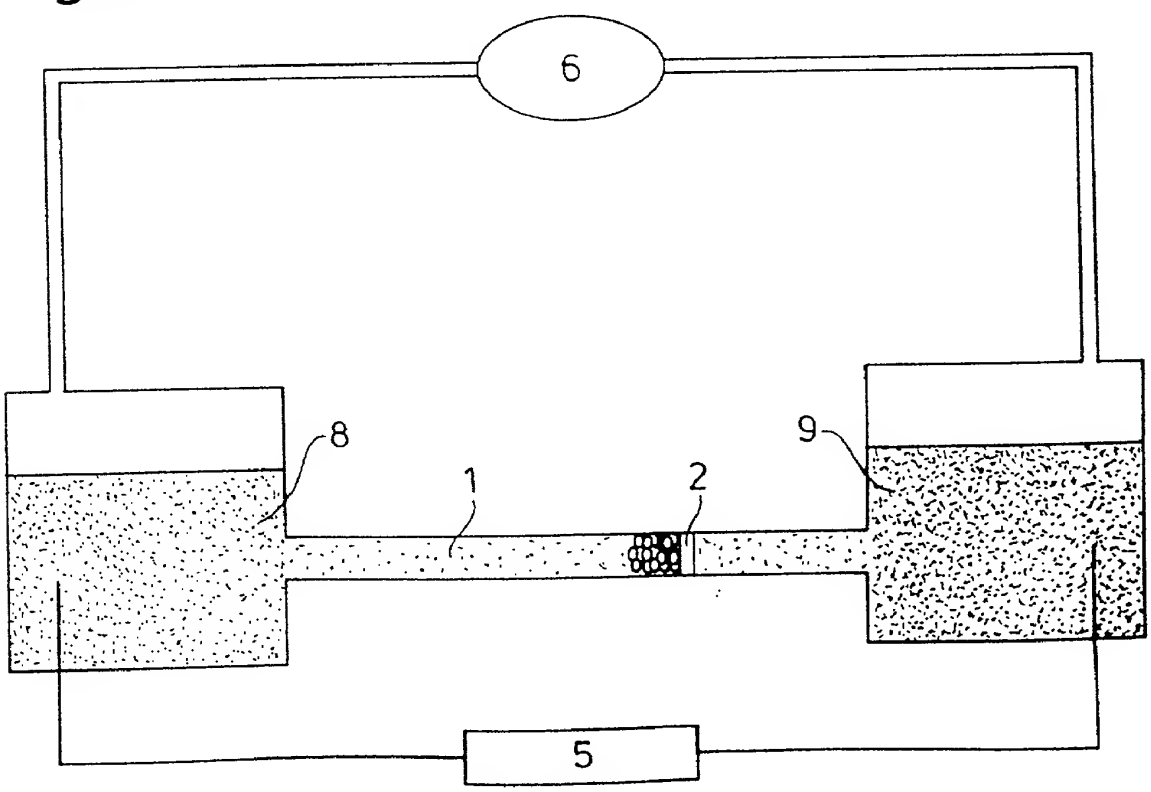


Fig. 4

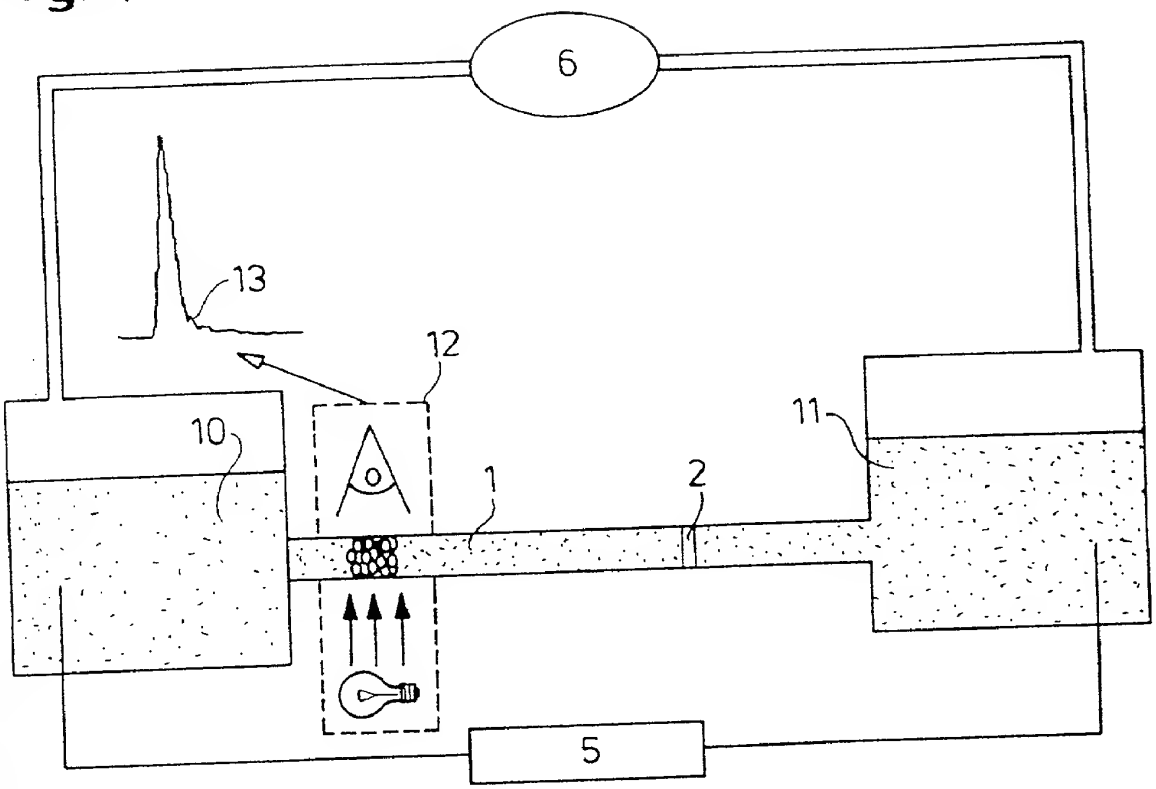


Fig. 5

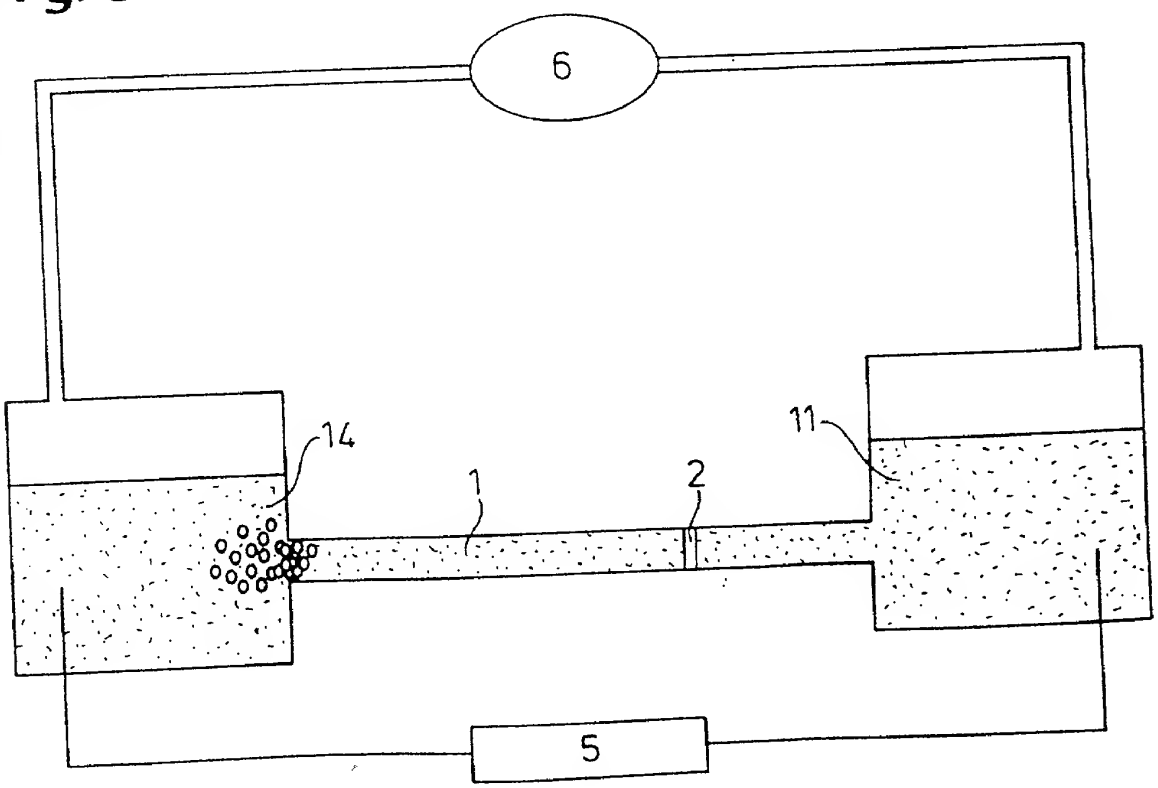


Fig. 6

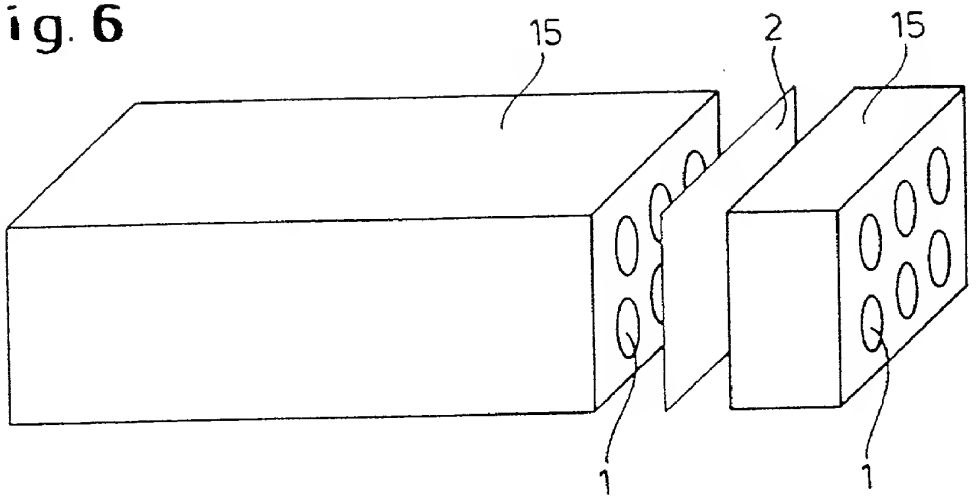
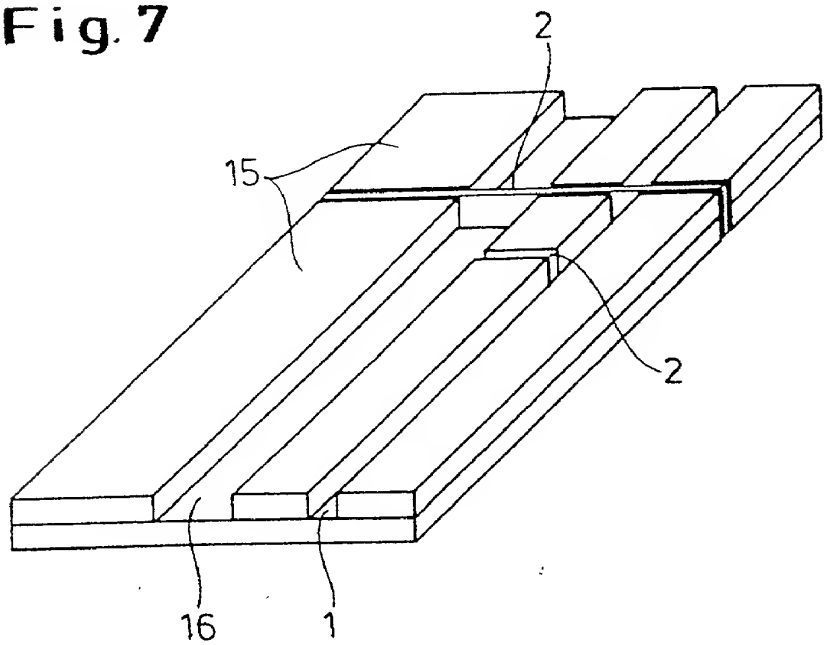


Fig. 7



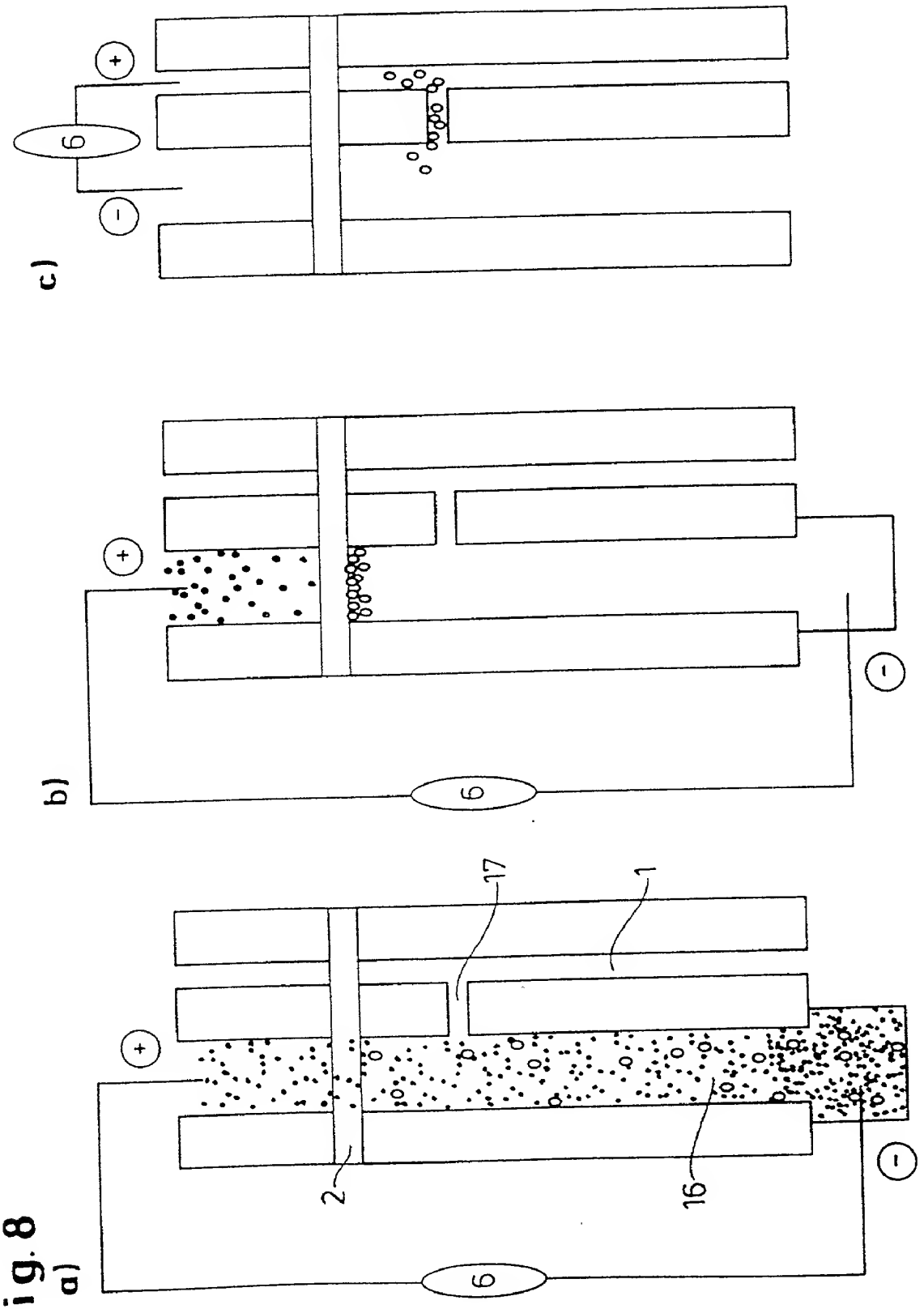


Fig.8

COMBINED DECLARATION AND POWER OF ATTORNEY	ATTORNEY DOCKET NO

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought
on the invention entitled

"ELECTROKINETIC SAMPLE PREPARATION"

the specification of which is attached hereto,

or was filed on **December 24, 1997**

as a PCT Application Serial No. **PCT/EP97/07306**

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s), the priority(ies) of which is/are to be claimed:

197 00 364.8	**Germany**	**January 8, 1997**
(Number)	(Country)	(Month/Day/Year Filed)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose the material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occured between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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